FORM PTO-1390 (REV. 11-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: GJE-73

U S APPLICATION NO (If known, see 37 CFR 1 5): 09/889256

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB00/00090	14 January 2000	15 January 1999

TITLE OF INVENTION
Pro-Apoptotic Agents

APPLICANT(S) FOR DO/EO/US
Sek Chuen Chow, David Idris Pritchard

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☑ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☑ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☑ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☑ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)) (unsigned).
10. ☐ An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☑ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 37 CFR 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
20. ☐ Other items or information:

U.S. APPLICATION NO (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
09/889256	PCT/GB00/00090	GJE-73

21. ☑ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =		
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$860.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total claims	5 - 20 =	0	x **$18.00**	$0.00	
Independent claims	4 - 3 =	0	x **$80.00**	$80.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$0.00	
TOTAL OF ABOVE CALCULATIONS =				$940.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +					
SUBTOTAL =				$940.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)).					
TOTAL NATIONAL FEE =				$940.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +					
TOTAL FEES ENCLOSED =				$940.00	
				Amount to be refunded:	$
				charged:	$

a. ☐ A check in the amount of $ _____ to cover the above fees is enclosed.

b. ☑ Please charge my Deposit Account No. 19-0065 in the amount of $ 940.00 to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☑ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0065 . A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

CORRESPONDENCE ADDRESS:

CUSTOMER NUMBER
23,557

David Saliwanchik
SIGNATURE

July 13, 2001
DATE

David R. Saliwanchik
NAME

31,794
REGISTRATION NUMBER

Patent Application
Docket No. GJE-73

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Sek Chuen Chow, David Idris Pritchard

Docket No. : GJE-73

For : Pro-Apoptotic Agents

Box PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF MAILING BY EXPRESS MAIL (37 CFR §1.10)

Express Mail No.: ET324078325US Date of Deposit: July 13, 2001

I hereby certify that the attached Transmittal Letter to the United States Designated/Elected Office (DO/EO/US); Preliminary Amendment; Declaration (37 CFR §1.63) and Power of Attorney; with copies as required for authorization for use of Deposit Account No. 19-0065, are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR §1.10 on the date indicated above and are addressed to: Box PCT, Assistant Commissioner for Patents, Washington, D.C. 20231.

ET 324078325 US
Name of person mailing paper

Signature

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PRELIMINARY AMENDMENT
Patent Application

July 13, 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Sek Chuen Chow, David Idris Pritchard

Docket No. : GJE-73

For : Pro-Apoptotic Agents

Box PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Please amend the above-identified patent application as follows:

In the Claims

The following amendments are made with respect to the claims in the international application PCT/GB00/00090.

Please substitute the following claim:

Claim 3 (amended):

A pro-apoptotic composition comprising a pharmaceutically acceptable diluent or carrier, and an excretory-secretory product, isolatable from *Necator americanus*, or a functional fragment thereof, capable of inducing apoptosis in reactive T-cells.

Please cancel claims 2, 4, and 5 and add the following new claims:

6. A method for treating cancer wherein said method comprises administering, to a mammal in need of such treatment, an effective amount of an excretory-secretory product isolatable from *Necator americanus*, or a functional fragment thereof, capable of inducing apoptosis in reactive T-cells.

7. The method, according to claim 6, wherein said mammal is a human.

8. A method for treating inflammation wherein said method comprises administering, to a mammal in need of such treatment, an effective amount of isolatable from *Necator americanus*, or a functional fragment thereof, capable of inducing apoptosis in reactive T-cells.

9. The method, according to claim 8, wherein said mammal is a human.

Remarks

By this Amendment claim 3 has been amended, claims 2, 4, and 5 have been canceled and new claims 6-9 have been added.

No new matter has been added by these amendments.

The Commissioner is hereby authorized to charge any fees under 37 CFR 1.16 or 1.17 as required by this paper to Deposit Account 19-0065.

Respectfully submitted,

David Saliwanchik

David R. Saliwanchik
Patent Attorney
Registration No. 31,794
Phone No.: 352-375-8100
Fax No.: 352-372-5800
Address: 2421 N.W. 41st Street, Suite A-1
Gainesville, FL 32606

DRS/la
Attachment: Marked-up Version of Amended claim

Marked-up Version of Claims

Claim 3 (amended):

A pro-apoptotic composition comprising a pharmaceutically acceptable diluent or carrier, and [a product as defined in either preceding claim] an excretory-secretory product, isolatable from *Necator americanus*, or a functional fragment thereof, capable of inducing apoptosis in reactive T-cells.

PRO-APOPTOTIC AGENTS

Field of the Invention

This invention relates to pro-apoptotic agents isolatable from Necator *americanus*.

Background to the Invention

Human nematodes (roundworms) include the hookworm nematode species, *Necator americanus*. Adult females of *N. americanus* are typically 9-11 mm in length and adult males are typically 7-9 mm in length. These adult worms commonly reside in the lumen of the small intestine, and attach to the intestinal wall resulting in blood loss from the host. Eggs are passed out in the faeces and, under favourable conditions, usually hatch in 1-2 days. Larvae are then released and continue to grow in the faeces and/or the soil. After up to 10 days, the larvae are infectious, and may survive 3-4 weeks in this condition. If, during this time, contact is made with a human host, the larvae can penetrate the skin, after which they may be carried through the veins and the heart to the lungs. Here, they penetrate the pulmonary alveolae and ascend the bronchial tree to the pharynx where they can be swallowed and delivered to the small intestine. They then develop into adult worms. Typically, six weeks or more is required from the initial infection to oviposition by the adult female.

N. americanus is found in tropical and sub-tropical localities, where it gives rise to a hookworm disease having a number of clinical features. Iron deficiency anaemia, resulting from blood loss at the site of intestinal attachment of the adult worms, is the most common symptom of hookworm infection, and may be accompanied by cardiac complications. Gastrointestinal and nutritional/metabolic symptoms may also be found. Additionally, itching may occur during the initial infection, and respiratory symptoms may be observed during the pulmonary migration stage.

Apoptosis is a suicide process built into all mammalian cells in which a cell dies in a controlled

manner. Cells undergoing apoptosis show distinctive morphological changes, for instance nuclear condensation and the formation of apoptotic bodies. The biochemical hallmark of apoptosis is the cleavage of chromatin into nucleosomal fragments.

Summary of the invention

The present invention is based on the realisation that hookworms shield against immunological attack by producing a factor capable of reducing the viability of reactive T cells. This factor may therefore exert an effect that results in cell apoptosis and may have valuable therapeutic application.

The present invention therefore provides a substantially pure excretory-secretory (ES) product, isolatable from *N. americanus*, and functional derivatives thereof, capable of reducing cell viability. Cell viability may be reduced via the induction of apoptosis.

The product of the invention may be a protein of less than 12kDa, or a functional fragment thereof.

The invention further provides a use for these ES products and derivatives, in the manufacture of a pro-apoptotic composition.

The invention further provides a pro-apoptotic composition comprising a pharmaceutically-acceptable diluent or carrier, and one or more ES product or derivative.

The invention further provides ES products or derivatives for use in the manufacture of a medicament with anti-tumour and/or anti-inflammatory activity, ie for the treatment of cancer or an inflammation disorder.

Brief Description of the Drawings

In the drawings:

Figure 1 shows the effect of *N. americanus* excretory-secretary products on the cell viability of human leukaemic T-cell line Jurkat, where (X) represents protein concentration (μg/ml) and (Y) represents the percentage cell viability; and

Figure 2 shows the effect of partially purified excretory-secretory products on the cell viability of the human leukaemic T-cell line Jurkat, where (X) represents cell fractions and (Y) represents the cell viability index.

Description of the Invention

By way of example only, excretory-secretory (ES) products of *N. americanus* may be prepared in the following manner.

Necator americanus is passaged in DSN hamsters. Faecal culture from the infected animals provide infective larvae, which are then used to infect neonates percutaneously. Adult worms are routinely harvested from the small intestine of infected hamsters 5 weeks post-infection. The ileum of the infected hamster is removed, opened longitudinally, and placed in Hanks' saline at 37°C. As worms release their hold on the mucosa, they are carefully removed, thoroughly washed, and cleansed in Hanks' saline containing 100 IU/ml penicillin and 100 μg/ml streptomycin. Cleansed worms are examined under a dissecting microscope, and undamaged worms retained.

Under sterile conditions, worms are added to RPMI 1640, containing penicillin and streptomycin, as above. The worms are then cultured for 16 hours, and the supernatants removed for analysis of pro-apoptotic activities.

Cultured supernatants are sterile-filtered through 0.2 μm filters, which also removes eggs that may have deposited during the culture period.

Protein concentration of the supernatants is assayed using Coomasie Brilliant Blue with BSA as standards.

To assess the effects of hookworm ES on the viability of Jurkat cells, 2 x 10^5 cells were cultured with various concentration of ES products in a final volume of 200 μl in flat-bottomed 96-well plates for 16 hours at 37°C in a 5% CO_2 incubator. This was followed by the addition of 20 μl of Thiazol blue solution (5 mg/ml) to the cells and the plates were incubated for a further 4 hours. After the

incubation, 150 µl of medium was removed carefully from the wells, followed by the addition of 150 µl iso-propanol, and mixed thoroughly. The OD at 590 and 650 nm was determined on an ELISA reader. Cell viability was expressed as the percentage of control absorbance obtained in untreated cells after subtracting the absorbance from appropriate blanks.

The induction of apoptosis in Jurkat T-cells by ES products was monitored by staining fixed cells with Hoechst dye 33358 (50 µg/ml in PBS) and examining the nuclear morphological changes using confocal laser microscopy, and the analysis of oligonucleosomal DNA fragments in the Jurkat cells using agarose gel electrophoresis.

Figure 1 shows the effect of *Necator americanus* ES products on Jurkat cell viability. Cell viability was reduced (ie cells were killed) in a dose-dependent manner. Cell viability was shown to be reduced via the induction of apoptosis. The characteristic cleavage of chromatin into nucleosomal fragments, that is indicative of apoptosis, was observed. A further characteristic of apoptosis is the change in nuclear morphology and this was also observed in the cells after treatment with ES products.

After fractionation through a Sephacryl S-300 column, the fractionated *N. americanus* preparation was assessed for pro-apoptotic activity. Each fraction was then co-cultured with Jurkat cells, and the cell viability index determined. Values of less than 1.0 indicate apoptotic cells. Figure 2 shows the cell viability index of fractions 1 to 45. Fractions 27-33 were found to have significantly lower cell viability indexes (<1.9) and therefore cell killing activities. Subsequent incubation of Jurkat cells with these fractions induced apoptosis in the cells. Fractions 27-33 were concentrated and separated on a 15% SDS PAGE. The gel showed very little protein bands but indicated that the pro-apoptotic agent may be less than 12 kDA in size.

The product of the invention may be formulated into a composition for therapeutic application. Suitable

formulations will be apparent to the skilled person, including acceptable excipients and diluents. The product may also be formulated with a carrier which targets a particular site *in vivo*, e.g. to a tumour.

CLAIMS

1. A substantially pure excretory-secretory product, isolatable from *Necator americanus*, or a functional fragment thereof, capable of inducing apoptosis in reactive T-cells.

2. A product according to claim 1, for use in therapy.

3. A pro-apoptotic composition comprising a pharmaceutically acceptable diluent or carrier, and a product as defined in either preceding claim.

4. The use of a product as defined in any preceding claim, in the manufacture of a medicament for the treatment of cancer.

5. The use of a product as defined in any of claims 1 to 3, in the manufacture of a medicament for the treatment of an inflammatory disease.

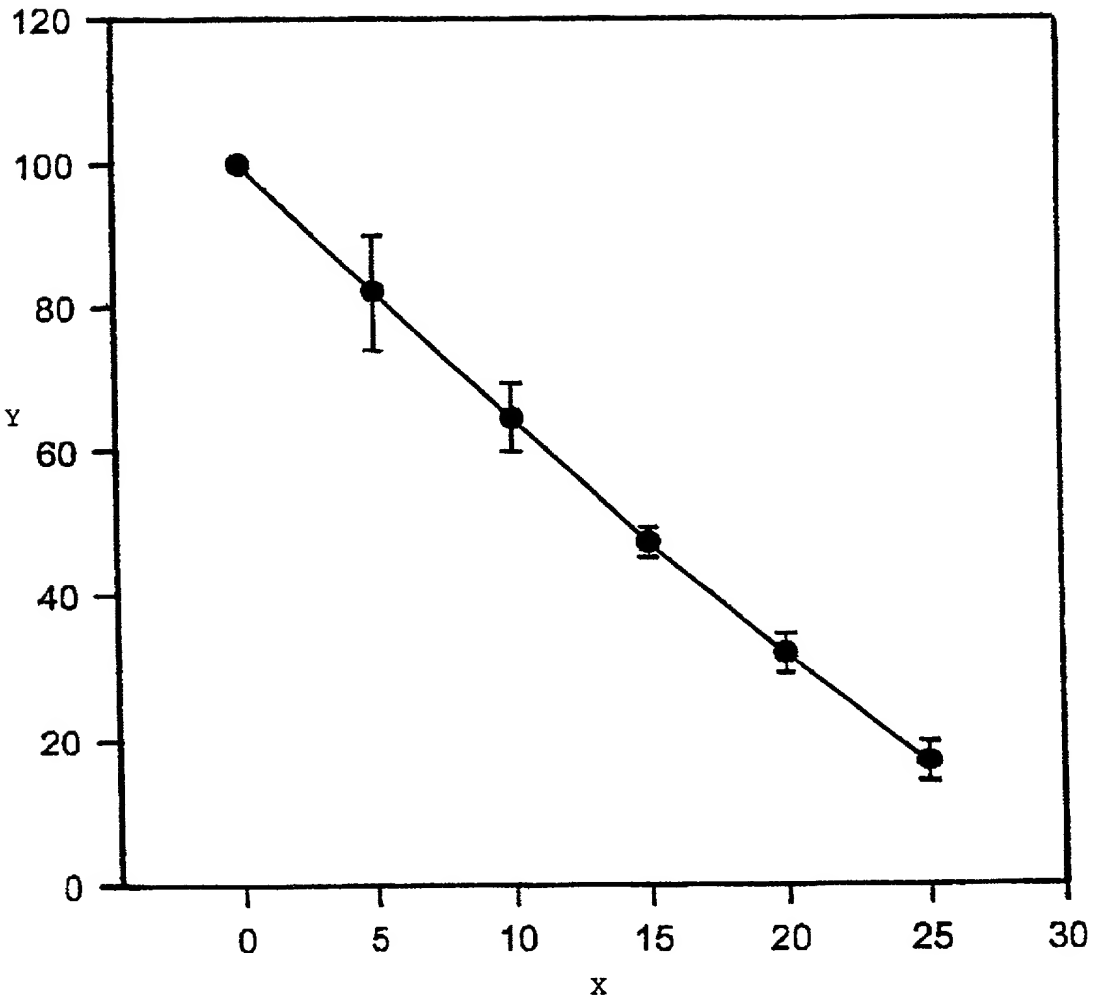

Figure 1

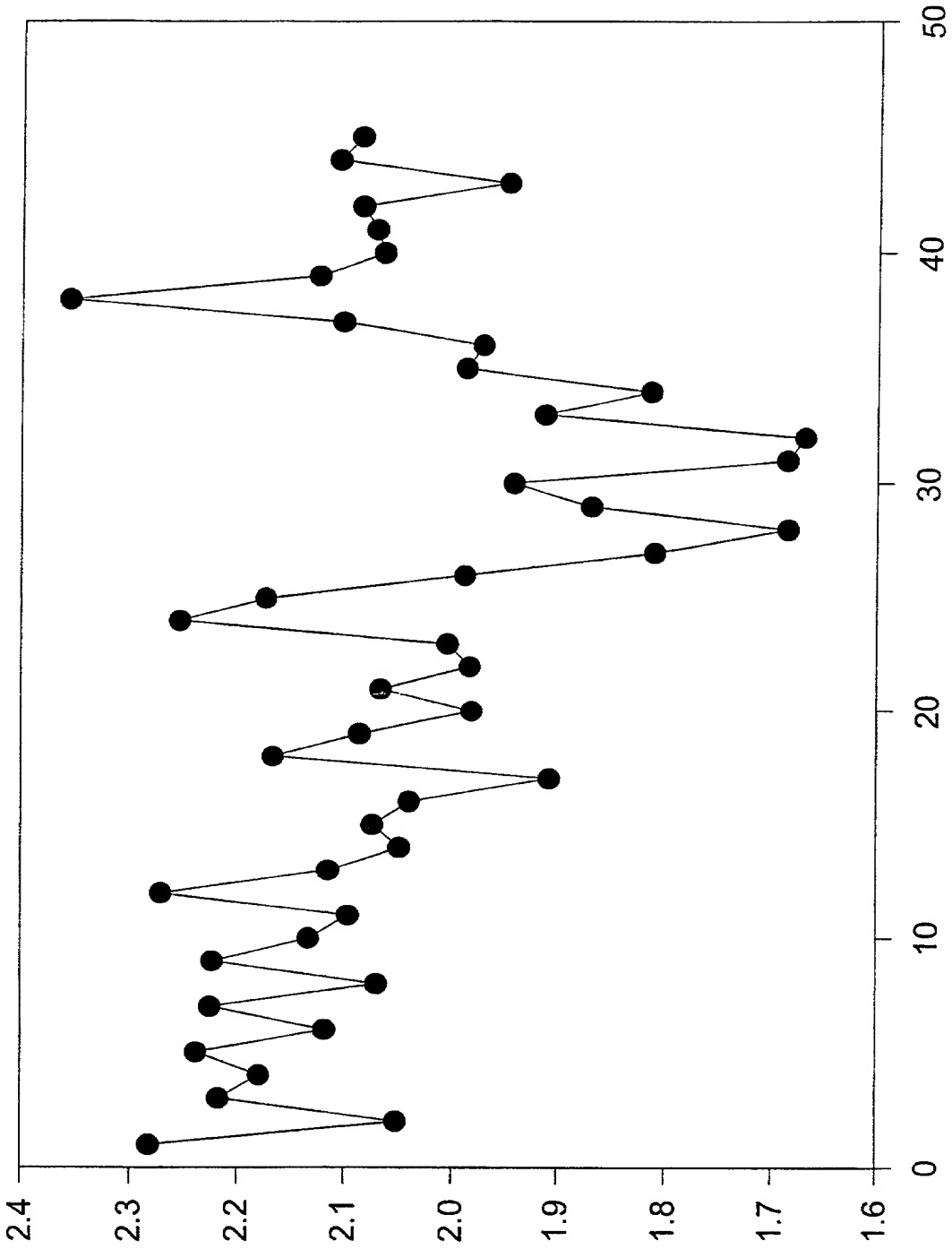

Figure 2

#3

USA

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name; I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of subject matter which is claimed and for which a patent is sought on an invention entitled

PRO-APOPTOTIC AGENTS

the specification of which ☐ is attached hereto or

☒ was filed on 14 JAN 2000 as United States Application Number or PCT International Application Number PCT/GB00/00090 and was amended on (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for a patent or inventor`s certificate, or PCT international application having a filing date before that of the application on which priority is claimed:

Prior Foreign Application Number(s)	Country	Foreign Filing Date	Priority Not Claimed	Certified Copy Attached? YES	NO
9900930.0	GB	15 JAN 1999	☐	☐	☐
			☐	☐	☐
			☐	☐	☐

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

(9) David R. Saliwanchik, Reg. 31,794; Jeff Lloyd, Reg. 35,589; Doran R. Pace, Reg. 38,261; Christine Q. McLeod, Reg. 36,213; Jay M. Sanders, Reg. 39,355; James S. Parker, Reg. 40,119 and Jean E. Kyle, Reg. 36,987; Frank C. Eisenschenk, Reg. 45,332; Seth M. Blum. Reg. 45,489

Direct all correspondence to:

Saliwanchik, Lloyd & Saliwanchik
2421 N.W. 41st Street, Suite A-1
Gainesville, FL 32606-6669
USA

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C 1001 and that such willful false statements may jeopardise the validity of the application or any patent issued thereon.

1-00

Full name of sole or First Inventor: Sek Chuen CHOW

Inventor's signature: _____

Residence address: Nottingham, United Kingdom GBX

Post Office address: University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom

Country of Citizenship: Malaysia Date of signature: 13/7/01

2-00

Full name of Second Inventor: David Idris PRITCHARD

Inventor's signature: _____

Residence address: Nottingham, United Kingdom GBX

Post Office address: University of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom

Country of Citizenship: United Kingdom Date of signature: July 16 2001